ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2005, p. 2126–2129 Vol. 49, No. 5
0066-4804/05/$08.00+0 doi:10.1128/AAC.49.5.2126–2129.2005

High Genetic Diversity of Ciprofloxacin-Nonsusceptible Isolates of *Streptococcus pneumoniae* in Poland

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Received 18 November 2004/Returned for modification 24 December 2004/Accepted 27 January 2005

We have analyzed the susceptibility to ciprofloxacin of 697 pneumococcal isolates collected in 1998–2002 in Poland from patients with respiratory tract diseases. Thirty-one ciprofloxacin-nonsusceptible isolates (MICs, ≥4 μg/ml) were identified, of which two were resistant to levofloxacin (MIC, 8 μg/ml). Serotyping, pulsed-field gel electrophoresis, multilocus sequence typing, and the analysis of resistance determinants showed their great genetic diversity.

The constant increase in resistance of *Streptococcus pneumoniae* to β-lactams, macrolides, and tetracyclines has evoked a need for alternative options in the treatment of pneumococcal infections. New fluoroquinolones, such as levofloxacin and moxifloxacin, are now considered to play this role in the case of infections in adults. However, the first pneumococci resistant to these compounds have appeared in some countries (5, 8, 9, 17, 19, 21, 32), and therapeutic failures have been reported (22). Mechanisms of quinolone resistance in *S. pneumoniae* include increased activity of the membrane pump PmrA (13) and modifications of the cellular drug targets topoisomerase IV (ParC/ParE) and DNA gyrase (GyrA/GyrB) (11, 18, 28, 29), located in their so-called quinolone-resistance-determining regions (QRDRs) (28, 29). Selection of these mechanisms is partially exerted by the common use of an older quinolone, ciprofloxacin, which is not recommended as an antipneumococcal agent. Each of the mechanisms alone confers low-level ciprofloxacin nonsusceptibility and increases the risk of acquisition of further changes (14). The accumulation of mutations in both ParC/ParE and GyrA/GyrB (3, 7, 18, 30, 32) results in high-level nonsusceptibility to ciprofloxacin and resistance to the newer compounds. Therefore, ciprofloxacin nonsusceptibility is an important measure of the actual and potential quinolone resistance of pneumococci (33).

The situation concerning resistance to quinolones in *S. pneumoniae* in Central and Eastern Europe has not been investigated yet. The aim of our study was to evaluate the frequency of ciprofloxacin nonsusceptibility in *S. pneumoniae* in Poland and to reveal the genetic relatedness among nonsusceptible isolates.

(This work was presented at RGPI-2, 10 to 12 December, 2004, Berlin, Germany.)

Six-hundred ninety-seven *S. pneumoniae* isolates were obtained from individual patients with lower respiratory tract diseases between 1998 and 2002 in 40 medical centers in 26 cities. The isolates were derived from sputum (562 isolates, 80.6%), bronchoalveolar lavage (75 isolates, 10.8%), and transtracheal aspirate (60 isolates, 8.6%). MICs of ciprofloxacin (Bayer AG, Leverkusen, Germany) were evaluated by the National Committee for Clinical Laboratory Standards microdilution method (26); due to the lack of an accepted breakpoint, a pneumococcal isolate was considered nonsusceptible to ciprofloxacin when its MIC was ≥4 μg/ml (1, 8, 17). Such isolates were tested as described above with levofloxacin (Aventis Pharma, Romainville, France), moxifloxacin (Bayer AG, Leverkusen, Germany), penicillin (Sigma Chemical Company, St. Louis, Mo.), and erythromycin (Fluka, Buchs, Switzerland), using the National Committee for Clinical Laboratory Standards-approved breakpoints (26). PCR amplification and sequencing of QRDRs of *gyrA*, *gyrB*, *parC*, and *parE* genes was performed as described by Pan et al. (29). The reserpine-mediated inhibition of quinolone efflux was performed according to the method of Broskey et al. (4). Serotypes of the isolates were determined by the capsular swelling method at the Statens Serum Institute (Copenhagen, Denmark). Pulsed-field gel electrophoresis (PFGE) typing was performed as described by Lefèvre et al. (23); isolates were considered indistinguishable when they shared PFGE patterns and were considered related when they showed a difference of one to three bands. Multilocus sequence typing (MLST) was performed as proposed by Enright and Spratt (10); the Internet-accessible database (http://www.mlst.net) was used to assign numbers to alleles and sequence types (STs).

Thirty-one isolates, i.e., 4.4% of the all 697 isolates studied (Table 1), appeared nonsusceptible to ciprofloxacin, and they originated from 12 towns uniformly distributed in the country. Among these isolates, five were penicillin nonsusceptible, two were erythromycin resistant and two (BY-2 and BY-3; 0.3%) were resistant to levofloxacin (MIC, 8 μg/ml) and intermediate to moxifloxacin (MIC, 2 μg/ml), which correlated with their high-level ciprofloxacin nonsusceptibility (MICs, ≥32 μg/ml). Both quinolone-resistant isolates were penicillin and erythromycin susceptible. No significant difference in patients' ages between the ciprofloxacin-nonsusceptible and -susceptible groups was found (56.7 ± 19.6 and 52.8 ± 21.2 years, respectively; $P = 0.3$). The prevalence of ciprofloxacin nonsusceptibility in *S. pneumoniae* is generally low worldwide; e.g., in the

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TABLE 1. Ciprofloxacin-nonsusceptible *S. pneumoniae* isolates: serotypes, susceptibility to quinolones, resistance determinants, PFGE types, and MLST characteristics

Isolate[a]	Serotype	MIC (μg/ml)[b]			Amino acid substitutions[c]				Efflux pump[d]	PFGE type	MLST allelic profile[e]	ST	Other isolation
		CIP	LVX	MOX	GyrA	GyrB	ParC	ParE					
WR-1/99	3	4	1	0.5	WT	WT	**D83N** K137N	WT	+	A	NP		
ML-2/02	3	4	1	0.12	WT	WT	WT	WT	ND	B	NP		
SU-1/98	6A	4	0.5	0.12	WT	WT	**S79F**	I460V	−	C	7-13-9-1-10-1-45	1363	New ST
SZ-1/98	6B	4	0.5	0.12	WT	WT	**S79F**	WT	+	D	8-13-14-4-1-4-14	200	Denmark, Taiwan
BY-8/01	6B	4	2	0.25	WT	WT	K137N	WT	+	E	7-25-4-2-48-20-28	497	Finland
BY-4/00	7C	4	0.5	0.12	WT	WT	WT	WT	+	F	NP		
BY-5/00	7C	4	0.5	0.12	WT	WT	**D83G**	WT	−	F	NP		
BY-2/98	7F	≥32	8	2	**S81F**	WT	**D83N** K137N	WT	−	G1	8-9-2-1-6-1-17	191	United Kingdom Denmark, Norway, Finland, The Netherlands, Uruguay, Brazil
BY-3/99	7F	≥32	8	2	**S81F**	WT	**D83N** K137N	WT	−	G2	8-9-2-1-6-1-17	191	
SA-1/99	9V	4	2	0.25	WT	WT	K137N	WT	+	H1	7-11-10-1-6-8-1	156	Spain^{9V}-3
BY-9/01	9V	4	2	0.25	WT	WT	**D83N** K137N	WT	−	H2	7-11-10-1-6-8-1	156	
SU-3/01	10A	4	2	0.25	WT	WT	**S79F**	I460V	+	J	NP		
SU-4/02	10A	4	1	0.25	WT	WT	**S79F**	I460V	+	J	NP		
SU-6/02	11A	4	1	0.12	WT	WT	**D83G**	WT	+	K	NP		
SA-2/02	14	4	1	0.12	WT	WT	K137N	WT	+	L	7-5-10-18-6-145-1	1477	New ST
BY-1/98	15A	4	0.5	0.12	WT	WT	**S79F**	WT	+	M	8-10-2-16-7-26-1	410	United Kingdom
Wpl-1/99	18C	4	0.5	0.12	WT	WT	**S79Y**	I460V	+	N	NP		
Wpl-2/99	18C	4	0.5	0.12	WT	WT	**S79F**	I460V	−	N	NP		
BY-10/02	19A	4	2	0.25	WT	WT	WT	I460V	+	O	18-5-9-1-47-1-6	482	Finland
SU-5/02	19A	4	0.5	0.12	WT	WT	WT	WT	+	P	2-19-2-17-6-22-14	276	The Netherlands
BY-6/01	19F	4	1	0.25	WT	WT	WT	I460V	+	Q1	1-5-4-12-5-3-8	423	United Kingdom
ML-1/01	19F	8	1	0.25	WT	WT	**S79F**	I460V	+	Q2	1-5-4-12-5-3-8	423	
KO-1/99	22F	4	0.5	0.12	WT	WT	**S79F**	I460V	−	R	NP		
LO-1/99	23B	4	0.5	0.12	WT	WT	WT	I460V	+	S	NP		
PR-1/99	23F	4	0.5	0.12	WT	WT	**S79F**	WT	−	T	7-5-1-1-13-31-14	440	United Kingdom
KR-1/99	23F	4	0.5	0.12	WT	WT	K137N	WT	+	U	7-8-8-18-10-6-14	1364	New ST
WR-2/00	23F	4	0.5	0.12	WT	WT	**S79F** K137N	WT	−	V	4-4-2-4-4-1-1	81	Spain^{23F}−1
Wpr-1/01	23F	4	2	0.25	WT	WT	WT	WT	+	W	15-17-4-16-6-19-17	239	United Kingdom
BY-7/01	23F	4	1	0.12	WT	WT	S52G K137N	WT	+	X	2-10-1-43-6-31-6	1014	New ST
SU-2/01	35F	4	1	0.12	WT	WT	WT	**P454S** I460V	−	Y	NP		
GD-1/01	37	4	1	0.12	WT	WT	WT	WT	ND	Z	NP		

[a] An isolate name contains an abbreviation of the center (BY, Bydgoszcz; GD, Gdańsk; KO, Kołobrzeg; KR, Kraków; LO, Łódź; ML, Mława; PR, Przemyśl; SA, Sanok; SU, Suwałki; SZ, Szczecin; Wpl, Warsaw 1; Wpr, Warsaw 2; WR, Wrocław), the sequential number of a resistant isolate from a given center, and the two last digits of the isolation year.
[b] CIP, ciprofloxacin; LVX, levofloxacin; MOX, moxifloxacin; WT, wild-type.
[c] Amino acid substitutions involved in resistance are shown in boldface type.
[d] ND, not determinable.
[e] NP, analysis not performed.

United States, it remained within the range of 1.2 to 1.6% during 1994-2000 (6). However, in some countries, such as Hong Kong, Ireland, and Spain, it has reached levels of 17.8%, 15.2%, and 5%, respectively (12, 15, 17). In Canada, the frequency of ciprofloxacin nonsusceptibility increased from 0% in 1993 to 1.7% in 1997-1998 following the increase in quinolone consumption (8). Therefore, while the observed rate of resistance to newer quinolones remains low in Poland (0.3%), the ciprofloxacin nonsusceptibility seems to be significant. Ciprofloxacin was introduced into the country in 1991; in 2002, its consumption in ambulatory care in Poland amounted to 0.5 defined daily doses/1,000 inhabitants/day, while in Spain it was 2.3-fold higher (16).

The reserpine-inhibited efflux was active in 19 ciprofloxacin-nonsusceptible isolates and absent in 10 isolates (Table 1). In 11 isolates, the efflux was the sole determinant of nonsusceptibility. Alterations in QRDRs of ParC/ParE or GyrA/GyrB were identified for 18 isolates, and they included predominantly single ParC mutations (15 isolates) at mutational hot spot Ser79 or Asp83 (29). Among them, the Ser79Phe substitution was the most common (10 isolates). A single isolate possessed the Pro454Ser substitution in ParE, which has been described before for clinical isolates (7, 9) and laboratory mutants (25). The role of some of the other observed substitutions is most probably negligible (3, 20, 31). The two levofloxacin-resistant isolates, in addition to the ParC mutation Asp83Asn, had the hot spot alteration Ser81Phe in GyrA (2). The proportions of frequency of the mechanisms of ciprofloxacin nonsusceptibility vary among countries; however, the alterations only in ParC/ParE seem to dominate (3, 4, 6, 11, 29, 30), reflecting the fact that ParC/ParE is a primary target for ciprofloxacin in pneumococcus (28, 29).

Eighteen serotypes were observed among the ciprofloxacin-nonsusceptible pneumococci, with the most common, 23F, being represented by five isolates (Table 1). Twenty-eight PFGE patterns were identified, and these could be classified into 25 distinct types. Three of the types (G, H, and Q) were differentiated further into two subtypes each, and one of these contained the levofloxacin-resistant isolates (type G). The results indicated the remarkable clonal diversity of ciprofloxacin-nonsusceptible *S. pneumoniae* in Poland, and suggested that they probably arose from multiple independent selection events. Such variability seems to be typical for the organism (24, 27), except in some countries, e.g., Spain, where clones Spain^{9V}-3 and Spain^{23F}-1 constitute 30% of ciprofloxacin-nonsusceptible pneumococci (1). Sixteen isolates, representing serotypes associated with the multiresistant international clones (6A, 6B, 9V, 14, 15A, 19A, 19F, and 23F), and the two levofloxacin-resistant isolates were subjected to MLST (Table 1). In general, the isolates were unrelated to the international clones; however, two and one isolates represented Spain^{9V}-3 (ST156) and Spain^{23F}-1 (ST81) clones, respectively. This observation is noteworthy, since the effective spread of such clones may quickly increase the rate of quinolone nonsusceptibility in a local pneumococcal population, as shown in Hong Kong (17). The levofloxacin-resistant isolates belonged to ST191, which was observed before in some European and South American countries (http://www.mlst.net).

In summary, the current frequency of ciprofloxacin-nonsusceptible pneumococci in Poland, although not alarming, is remarkable. The circulation of strains that are prone to develop resistance also to newer quinolones may compromise this therapeutic option in the future and undoubtedly requires permanent epidemiological surveillance.

We thank Paweł Grzesiowski for help with statistical calculations and Anna Klarowicz and Agnieszka Mrówka for their technical assistance.

This study was partially financed by a grant from the Polish Committee for Scientific Research (3P0A 062 23). We acknowledge the use of the pneumococcal MLST database which is located at Imperial College, London, and is funded by the Wellcome Trust.

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